REMARKS

Claims 1 -53 are pending in the application. Claims 17-23, 26 and 45-49 have been previously withdrawn from consideration. Claims 54-73 have been previously cancelled. Claims 1 and 4 are currently amended.

Claims 1 and 4 are amended for purpose of clarification. Claim 1 is amended to recite that the grain protein in the formulation has substantially no heat denaturation. Support for this amendment can be found in paragraph 12 on page 3, paragraph 9 on page 2, and paragraph 28 on page 7 of the Specification as originally filed. Claim 4 is amended to correct a typographical error.

I. Claim Rejections under 35 U.S.C. §103(a) over Wang in view of Kitabatake.

Claims 1-5, 7-16, 24, 25, 27-33, 41, 42 and 54 stand rejected under 35 U.S.C. §103(a) as being unpatentable over PCT Publication No. WO 00/13521 to Wang *et al.* (hereinafter "Wang") with evidence from Kitabatake et al., Agri. Biol. Chem., 54(9) p2205, 1990 (hereinafter "Kitabatake"). Applicant respectfully disagrees with this rejection. Applicant also observes that Kitabatake is not prior art but is only cited by the Examiner as evidence to support the Examiner's argument.

Obviousness is a question of law based on underlying factual inquiries. The factual inquiries (also known as the "Graham factual inquiries") to be performed by the Examiner are as follows:

(1) Determining the scope and content of the prior art;

(2) Ascertaining the differences between the claimed invention and the prior art; and

(3) Resolving the level of ordinary skill in the pertinent art.

Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in KSR International Co. v. Teleflex Inc., Federal Register, Vol. 72, No. 195, 57526-35, 57526 (October 10, 2007). Once the Graham factual inquiries are resolved, the Examiner must determine whether the claimed invention would have been obvious to one of ordinary skill in the art. Prior art is not limited just to the references being applied, but includes the understanding of one of

ordinary skill in the art. Although the prior art reference (or references when combined) need not teach or suggest all the claim limitations, the Examiner must explain why the difference(s) between the prior art and the claimed invention would have been obvious to one of ordinary skill in the art. *Id.* 57528.

The Supreme Court noted in the *KSR* case that the analysis supporting a rejection under 35 U.S.C. 103 should be made explicit. The Court stated that "'rejections on obviousness cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness.'" *KSR International Co. v. Teleflex Inc.*, 127 S. Ct. 1727 at 1741, 82 USPQ2d 1385 at 1396 (2007), quoting *In re Kahn*, 441 F.3d 977, 988 (C.A.Fed.2006). The Court also reiterated the long-held tenet against a "temptation to read into the prior art the teachings of the invention in issue" and "against slipping into the use of hindsight." Id., at 1742, quoting *Monroe Auto Equipment Co. v. Heckethorn Mfg. & Supply Co.*, 332 F.2d 406, 412 (C.A.6 1964).

Wang discloses a chewable pet toy made from a protein-based thermoplastic composition containing plant or animal derived proteinaceous material and various additive and nutritional ingredients. However, Wang neither teaches nor suggests Applicant's specific teachings to avoid substantial heat denaturation of the grain protein as is now claimed by Applicant. As the Examiner states, in discussing the teachings of Wang, "there is no restriction on the temperature to be used other than providing a product having good flow when used in preparing the injected molded article." Lines 6-7, page 3 of the Office Action dated March 24, 2008. Applicant agrees. There is no express or implied limitation in Wang to avoid substantial heat denaturation.

The Examiner reasoned that because Wang's composition has a moisture content of as low as 10%, the protein in Wang's composition is necessarily undenatured. In support of this notion, the Examiner cited Kitabatake as evidence to show that the denaturing temperature of a protein increases as the moisture content of the protein decreases. However, the Examiner fails to consider the effects of other ingredients, such as plasticizers, on the denaturing temperature of the protein in Wang's composition. Claim 1 of Wang recites 10-30% of water, and 0-30% edible plasticizer without specifying the relationship between the water content and the content of the plasticizer.

Wang states that "[t]ypical edible plasticizers which can be used in the present invention include glycerol, sorbitan, ethylene glycol, propylene glycol, diethylene glycol, dipropylene glycol, mannitol, and sorbitol." See lines 25-28, page 7 of Wang.

Because Wang does not specify what combination of water content and plasticizer content would work for the invention disclosed therein, the Specification of Wang needs to be consulted in order to determine the contents of water and plasticizer as taught by Wang. Example 1 and Figure 2 of Wang show the relationship between water content and the content of the plasticizer. In Figure 2, the two curves, I and II, define three regions, A, B and C. According to Wang, "[i]n region B, the soy protein isolate composition was easy to flow in injection molding processing conditions. In region C, the soy protein isolate composition was hard to flow and showed thermoset behavior. In region A, the soy protein isolate composition flowed better and showed thermoplastic behavior." Page 10 of Wang. Wang also discloses that "[t]he present protein-based molding composition has good processing flowability...." Lines 22-23, Page 3 of Wang. Taken together, in order for the composition of Wang to have good processing flowability, the contents of water and plasticizer should at least fall into Regions A or B of Figure 2, but not C. As shown in Figure 2, when the water content is about 10%, the plasticizer needs to be present at a minimum of about 30% to maintain good processing flowability. Thus, one of ordinary skill in the art would conclude that when the water content is about 10%, a plasticizer content of at least about 30% is required to practice the invention of Wang.

Although Kitabatake has not addressed the effects of edible plasticizers on the denaturing temperature of a grain protein, other references have demonstrated that denaturation of a protein depends on factors such as temperature, presence or absence of denaturants, plasticizer, as well as interfaces. See e.g., page 302, left col., lines 24-29, De Graaf, J. of Biotech., 79: 299-306 (2000) (enclosed as Appendix A). Indeed, De Graaf shows that the presence of plasticizer may lower the Glass transition temperature T_G, thus decreasing the threshold for protein denaturation. Id., page 302, right col., lines 23-29; page 303, left col., lines 8-12. Thus, the effect of relatively low water content may have been counter-balanced by the addition of edible plasticizers to the Wang composition. Based on the combined teaching of Kitabatake and De Graaf, it is speculative for the

Examiner to conclude that the protein in Wang's composition is substantially undenatured.

The Examiner also reasoned that "even if the moisture higher, it is expected that the speed of treatment in during (sic) extrusion would not allow for significant denaturation to occur, thus providing a product that is substantially undenatured." Page 3 of the Office Action. Here, the Examiner used a conclusory statement in place of reasoning to support an obviousness rejection. Applicant disagrees with the Examiner's position because denaturation of protein may happen within a very short period of time. As shown by Weijers et al., heat denaturation of ovalbumin have a time of about 1.2 minutes at 80 degree, a temperature that is much lower than that used by Wang. See Table 1, page 2697 of Weijers et al., Protein Science, 12:2693-2703 (2003) (enclosed as Appendix B). Applicant realizes that grain proteins are different from ovalbumin, but is citing the Weijers paper merely to rebut the Examiner's position that denaturation of a protein does not happen if the protein is only exposed to the denaturing temperature for a very short period of time. If the Examiner insists that the protein contained in Wang's composition is substantially undenatured by heat, Applicant respectfully requests supporting evidence for the record.

Claims 2-5, 7-16, 24, 25, 27-33, 41 and 42 depend from claim 1, and benefit from arguments presented above. Further, these claims contain additional features that patentably distinguish over Wang. For example, claims 7-9 recite specific amounts of hydrolyzed protein suitable for use in the resin formulations. Wang does not disclose a particular amount or range of hydrolyzed protein. Claims 10-16 relate to hydrolyzed protein derivatives. Wang fails to disclose hydrolyzed protein derivatives. In particular, claim 11 recites a hydrolyzed protein derivative comprising a reaction product of a protein hydrolyzate with at least one reagent selected from the group consisting of an anhydride, ethylene oxide, propylene oxide, fatty acid, reducing sugars, maltodextrin, oligosaccharide, and dextrin. Wang is silent as to reactions involving protein hydrolyzates. Claims 12 and 13 depend directly from claim 11, and benefit from like argument.

Taken together, because significant differences exist between the cited references and Applicant's claimed invention, and because the Examiner has not carried the burden

of showing with some reasoning that such differences would have been obvious to one of ordinary skill in the art at the time of Applicant's invention, withdrawal of the §103 rejections over Wang with Kitabatake as evidence is respectfully requested.

II. Claim Rejections under 35 U.S.C. § 103(a) over Wang and Axelrod.

Claim 6 stands rejected under 35 U.S.C. §103(a) as being unpatentable over Wang and U.S. Patent No. 6,159,516 granted to Axelrod *et al.* (hereinafter "Axelrod"). Applicant respectfully disagrees. Claim 6 depends indirectly from claim 1, and benefits from arguments presented above in Section I. Axelrod discloses a process for forming starch into a molded article using melt processing techniques and does not cure the defects of Wang as discussed in Section I. Withdrawal of the §103 rejections of Claim 6 is respectfully requested.

III. Claim Rejections under 35 U.S.C. § 103(a) over Wang and Pater.

Claims 34-37 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Wang and U.S. Patent Application Publication No. 2003/0219516 to Pater *et al.* (hereinafter "Pater"). Applicant respectfully disagrees. Claims 34-37 depend directly or indirectly from claim 1, and benefits from the arguments presented above in Section I. Pater discloses a pet chew based on starch that may be native or chemically modified, e.g., oxidized, carboxymethylated, hydroxyalkylated, acetylated, or (partially) hydrolyzed, and does not cure the defects of Wang as discussed in Section I. Withdrawal of the §103 rejections of Claims 34-37 is respectfully requested.

IV. Claim Rejections under 35 U.S.C. § 103(a) over Wang and Jane.

Claims 34-36, 38-44 and 50-53 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Wang and U.S. Patent No. 5,523,293 granted to Jane *et al.* (hereinafter "Jane"). Applicant respectfully disagrees. Claims 34-36, 38-44 and 50-53 depend directly or indirectly from claim 1, and benefit from the arguments presented above in Section I. Jane discloses biodegradable, thermoplastic compositions made from the reaction product of soybean protein, a carbohydrate filler, a reducing agent, a plasticizer, water and optional additives, and does not cure the defects of Wang as discussed in Section I. Withdrawal of the §103 rejections of Claims 34-36, 38-44 and 50-53 is respectfully requested.

CONCLUSION

In view of the foregoing reasons, Applicant has addressed all issues raised in the Office Action dated March 24, 2008, and respectfully solicits a Notice of Allowance. Should any issues remain, the Examiner is encouraged to telephone the undersigned attorney.

Authorization to charge fees associated the Petition for three-month extension of time is submitted herewith. If any additional fee is deemed necessary in connection with this Response, please charge Deposit Account No. 12–0600.

Respectfully submitted,

LATHROP & GAGE LC

Date: September 24, 2008

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Appendix A

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Denaturation of proteins from a non-food perspective

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Abstract

Controlled denaturation, or the prevention of denaturation, is an important aspect in the development of food applications of proteins. For the use of proteins in non-food applications such as surfactants, adhesives, coatings or plastics, it is discussed that a certain degree of denaturation must occur in order to make proteins processable, and to reach the required product properties such as strength, water resistance or adhesion. By adjusting the processing parameters (temperature, water content, chemicals) conditions can be created to allow structural changes in the protein. The effect of processing on some product properties will be discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Proteins; Denaturation; Glass transition; Processing; Properties

1. Introduction

Industrial proteins are a class of renewable materials that are produced at a kiloton scale per annum. Proteins can be derived from plants, e.g. wheat gluten, soy and pea protein, or from the milk or skin and hides of animals, such as casein and whey protein, and gelatin respectively. Currently a number of industrial applications is based on industrial proteins (Skeist, 1990; Mulder, 1997; Sander et al., 1997). Casein is still used in labelling adhesives, and gelatin finds application in hot melts in bookbinding and as a stabiliser/binder in photographic emulsions (with non-surpassed performance). Soy protein is applied as paper sizing agent and plywood adhesive. A more recent development is the use of (plant) proteins in surfactants and cosmetics.

Non-food protein research is currently more driven by a market-pull from consumers than by a technology-push from protein producers. It becomes clear that proteins have unique properties that can be exploited for several applications (Kolster et al., 1997; De Graaf and Kolster, 1998; De Graaf et al., 1998; Lens et al., 1999b).

Though for food applications it is important that the proteins are in the native (not denaturated) state in order to exert the desired functionality, for technical applications this is less relevant. In this paper, it will be shown that in many cases, denaturation is a prerequisite for the processing of proteins into a product and for

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achieving a good product performance. Though denaturation is a prerequisite, to what extent protein denaturation, or structural changes in general, are necessary (or allowed from a properties point of view) or what influence denaturation has on the final product properties are questions to which the answer is mostly unknown as yet.

The paper will discuss why protein denaturation is important for technical applications. In most cases, denaturation occurs during processing and it often is a prerequisite for obtaining the desired properties of the final product. Subsequently, protein denaturation is qualitatively discussed, and combined with a very important aspect: the molecular mobility of the protein material as required for denaturation to occur. Structural changes that can take place during protein processing are discussed, and a hypothesis for film formation from (non-water soluble) proteins is given. Finally, some examples are given on the influence of processing on the properties of protein films/coatings.

2. Industrial proteins for technical applications: physicochemical requirements and processing methods

For most technical applications, denaturation is required in order to reach the desired properties. First an overview is given the physico–chemical properties of proteins which are necessary for, for instance, adhesion or film formation. Subsequently, some common processing procedures for the formation of coatings, adhesives and plastics are discussed. Most procedures use a combination of water, denaturants and increased temperature, which all favour denaturation.

2.1. Physico–chemical requirements for proteins in technical applications

Table 1 gives an overview of a number of technical applications of proteins, and the requirements for good product performance. Examples are the good adhesion and bond strength for adhesives, resistance against water for coatings and strength for plastic materials. The routes to obtain a good performance are added in the last column. Adhesion is reached upon exposure of specific groups (e.g. polar groups onto polar surfaces such as glass and metals and apolar groups onto apolar surfaces such as most plastics). In order to obtain a high cohesive strength, molecules have to be entangled. The cohesive strength and water resistance can be enhanced by crosslinking of the protein polymers for which reactivity is a prerequisite. Reactivity implies exposure of reactive (polar) groups such as acid, amino, hydroxyl and sulphydryl groups. Exposure

Table 1
Physico-chemical requirements for some technical products

Product	Example	Property	Obtained by
Coating	Paint, ink	Adhesion	→ exposure specific groups
	Paper, packaging	Film strength	→ entanglements
	coating	Water resistance	→ crosslinking (reactive groups)
Adhesive	Water-based	Processability	→ dissolution
	Hot melt	Tack (adhesion)	→ exposure specific groups
		Bond strength	→ entanglements
		Water resistance	→ crosslinking
Plastic	Disposable, fiber,	Melt strength	→ entanglements
	Foil/packaging	Tensile strength	→ entanglements
	coating, laminates	Water resistance	→ crosslinking
Surfactants	Emulsifier,	Surface tension	→ exposure specific groups
	detergent, wetting agent	Stabilisation of interface	→ exposure specific groups

of specific groups and the formation of entanglements (physically entangled chains) imply that the protein has become less structured or even denatured. The decrease in water uptake of proteins, and thereby the dependence of properties on the water content, is an important aspect of research on technical applications of proteins. It was shown that denaturation significantly decreases the water uptake of ovalbumin (Kumagai et al., 1997). Though this phenomenon was thoroughly investigated for ovalbumin, it might be common for proteins. To conclude, Table 1 shows that in order to obtain good products, a certain extent of protein denaturation (structural changes) must occur.

2.2. Common processing procedures for technical products

Proteins can be processed in the presence of a high amount of water (e.g. coatings, adhesives, surfactants), or under low-moisture conditions (extrusion). Generally, protein based coatings and adhesives are produced by dissolving the protein in water at high or low pH and/or using denaturants such as urea (Skeist, 1990; Somanathan et al., 1992; Gennadios et al., 1994; McHugh et al., 1994). Often the temperature is increased to facilitate dissolution. In most cases the dry solids matter of the solution does not exceed 20% w/w. These solutions can be cast into coatings, or formulated into adhesives by means of urea and borax (Skeist, 1990). Alternatively, it was shown that by dispersing a non-water soluble protein such as wheat gluten in water at neutral pH, also strong flexible films could be prepared (Lens et al., 1999a).

Surfactants usually are comparatively small molecules (molecular weight (far) below 40 000) which tend to accumulate at interfaces and stabilise these interfaces.

Well-known protein surfactants are casein, gelatin and recently also plant based surfactants which are used in personal products (e.g. shaving foams, creams). The remainder of this text will focus on medium and higher molecular weight proteins.

Thermoplastic materials are processed by means of extrusion. Dry powder is fed into an extruder with a sufficiently high amount of water (usually > 20%), other plasticizers such as glycerol, and/or other additives (Camire, 1991; Tolstoguzov, 1993). The material is intensively mixed at temperatures of 100–200°C, and molten into a dough-like texture. After mixing, the product is shaped and the molecular structure that was formed during extrusion is fixed. This process will be discussed in more detail in a next paragraph.

The important feature of both the wet processing into adhesives, coatings and surfactants and the 'dry' processing into plastics is that in all cases a combination is used of a high amount of water, denaturants and increased temperature. These conditions induce denaturation, as will be shown in the next paragraph. Moreover, denaturation seems to be a prerequisite for most processing methods, e.g. for the solubilization of the protein for coatings and adhesives, or the formation of a plastic mass during extrusion.

3. Importance of molecular mobility on denaturation

3.1. Protein denaturation

Protein denaturation is the unfolding of the protein from a structured native state into an (partially) unstructured state with no or little fixed residual structure, which is not far from a random coil. Denaturation can be induced both by temperature and by denaturants (chemical denaturation). Above medium water content (> 5%) and above temperatures of circa 75°C denaturation occurs. However, it is generally known that the denaturation temperature of proteins may differ due to the protein source, to additives (denaturants such as urea or guanidinium HCl) and processing methods. Moreover, denaturation can be reversible or irreversible.

Though the pathways of thermal and chemical denaturation are different, the final result is the same (Fujita and Noda, 1981). So far, virtually all information on protein denaturation was obtained from research on solutions with a water content

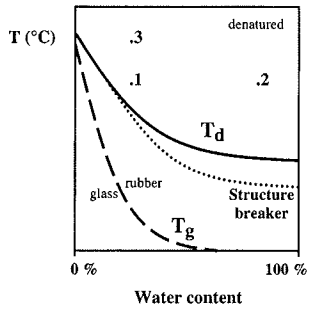

Fig. 1. Dependence of denaturation temperature (T_D, solid line) and glass transition temperature (T_G, dashed line) of proteins on the water content. The influence of structure breakers on T_D is shown (dotted line). Three processing conditions are depicted in points 1–3 (drawn after Luescher et al., 1974; Sochova, 1997).

> 90%, and little research has been performed on denaturation in systems at low or medium water content. Though studies in dilute solutions are often considered to be representative for the more concentrated state, these studies do not necessarily reflect the structure in solid state (Shewry et al., 1994).

3.2. Denaturation temperature T_D: a thermodynamic phenomenon

The denaturation temperature of the protein, T_D, strongly depends on the water content up to a water content of ≈10–20% (see Fig. 1, drawn after Luescher et al., 1974; Fujita and Noda, 1981; Kitabake et al., 1989; Sochova 1997). Generally T_D decreases from 120 to 200°C down to 80°C between 0 and 20% w/w water. Luescher describes that in the case of tropocollagen, the influence of the water content on T_D by thermal denaturation could be decreased by adding a structure stabiliser (e.g. potassium fluoride), while the influence was enhanced by adding a structure breaker (potassium iodide).

Denaturation as described in most literature references is a purely thermodynamic phenomenon which depends on temperature (i.e. energy of interactions), but also factors such as the presence of denaturants and plasticizers (e.g. water) and interfaces. However, in order for thermodynamic phenomena to take place in a material on a measurable time-scale, the molecular mobility of the material should be sufficient, that is, above the glass transition temperature.

Glass transition temperature T_G: molecular mobility proteins, as high molecular weight materials, show a glass transition temperature T_G (Hoseney et al., 1986; Kalichevsky et al., 1992; Sochova and Smirnova, 1993; Sochova, 1997). T_G is the onset temperature of coordinated molecular mobility in the polymer main chain, from the glassy (immobile) to the rubbery (mobile) state (Sperling, 1990). Below T_G, only a maximum of four chain atoms are involved in motions. In the glass transition region 10–50 chain atoms attain sufficient thermal energy to move in a coordinated (crankshaft) manner. Above T_G, large chain segments are able to move and the material is mobile. As proteins consist of over 20 amino acid monomer units, and therefore their chain structure is quite heterogeneous, the concept of T_G is probably a simplification but suitable for this discussion.

T_G depends on the molecular structure such as the rigidity of main chain and side-chains and on crosslinks, and also on the presence of plasticizers. Plasticizers are low molecular weight substances that dissolve in the polymer, thereby increasing the free volume and mobility of the polymer. This results in a lowering of T_G. In the case of proteins, water and glycerol are the most well-known plasticizers. The dependence of the protein T_G on plasticizers such as water is added in Fig. 1. It can be observed that the dependence of T_G on the water content is stronger than that of T_D on the water content: T_G decreases more rapidly than T_D upon increasing water content. T_G usually is room temperature at a total plasticizer content of about 25%.

3.3. Molecular mobility and denaturation

The principles of denaturation and glass transition were combined by Sochova, who states that the heat capacity jump at T_G and denaturation of globular proteins are essentially based on the same physical process: an increase of conformational mobility in the globules (Sochova and Smirnova, 1993; Sochova, 1997). This means that

protein denaturation, or structure changes in general, can only occur if the mobility of the material is sufficiently high and $T_D > T_G$. This view differs from the generally accepted views of its hydrophobic origin of denaturation (Privalov and Gill, 1989; Privalov, 1990). This paper will not go into the specifics of both hypotheses.

Thus, in order to denature a protein, the system should be sufficiently above both the T_D and T_G-water content line in Fig. 1, allowing both thermodynamic denaturation ($T > T_D$) and sufficient chain mobility ($T > T_G$). Concerning processing this can be achieved by:

- increasing the water content, allowing processing at 'low' temperature (shift from point 1 to 2 in Fig. 1);
- increasing the temperature, which is particularly effective at low water content (shift from point 1 to 3 in Fig. 1);
- addition of structure breakers/denaturants which reduce T_D at a given water content (e.g. urea).

During common processing procedures of turning materials into coatings (aqueous solutions or dispersions), adhesives (urea-containing solutions) and during extrusion (increased temperature, water added) these conditions generally are met. However, the degree of denaturation (or structural changes in general) may differ between proteins and processing methods.

4. Structure changes during protein processing

In the last paragraphs it was discussed that both for the processing of proteins into coatings, adhesives and extruded materials, and for reaching the final properties of the products, a certain amount of structure changes must occur in the protein. In general, three stages can be distinguished during processing (Tolstoguzov, 1993) and especially in the first stage structure changes will occur.

4.1. Mixing

Solubilization/dispersion of the protein in water or other solvents, or mixing protein powder with water/plasticizers. Increased temperature, shear and the presence of denaturants are quite common in this stage. Molecular interactions are disrupted, sulphur bridges may be broken and structure changes do occur. For instance, Wellner showed that the hydration of ω-gliadins initially resulted in the formation of β-sheets which disappeared upon subsequent increase in water content of the sample (Wellner et al., 1996). Weegels et al. (1994) showed that the secondary structure of wheat gluten is changed upon heating to 80°C in the presence of water. The water content determined whether the gliadin fraction was most affected (at water contents < 10%) or the glutenin fraction (water content > 21%). In both cases the α-helix content was significantly decreased.

4.2. Structure formation

In the second stage, molecules will be newly arranged. In the case of extruded samples, the shearing conditions favour alignment of the molecules, leading to new interactions (Camire, 1991). Fibrillar proteins were shown to change from α-helix into β-sheets during extrusion (Yuryev et al., 1990).

During coating and adhesive processing, denaturation occurs at the air-dispersion and dispersion-substrate interfaces. Film structure may differ from the solution structure as was shown, for instance, during film formation of glycinin, where a substantial increase in the β-sheet content was observed in the dried film (Subirade et al., 1998).

4.3. Structure fixation

After molecular orientation and shaping of the product, any structure formed has to be fixed. Fixation can be physical or chemical or a combination of the two. Physical fixation, or vitrification, is the reduction of molecular mobility by decreasing temperature to below T_G (e.g. after extrusion), or decreasing water content (drying of coatings and adhesives). Formation of β-sheets is a very effective fixation mechanism.

Chemical fixation is for instance the formation of sulphur-bridges or of covalent linkages between

non-sulphur reactive groups in the protein, of which the former is by far the most important (Prudencio-Ferreira and Areas, 1993; Ledward and Tester, 1994; Li and Lee, 1997). Another possibility is to fixate the structure by means of added crosslinkers, thereby increasing for instance the strength or water resistance of the material (De Graaf and Kolster, 1998).

4.4. Protein film formation

The formation of protein films, both from solution and suspension, differs from the formation of films from dispersions of polymer particles. Non-water soluble polymer particles show interdiffusion of molecules in the outer layer of the particles, and the particles do not change shape (Verkholantsev, 1998). Upon electron microscopic investigation of wheat gluten films in our institute, no residual particulate structure could be observed, though the suspensions showed particles of at the highest 250 μm size.

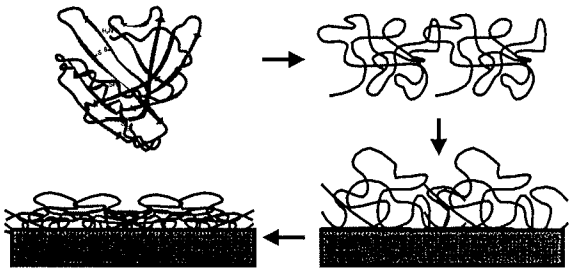

Fig. 2. Schematic representation of film formation from a protein solution or suspension. Structure changes may occur during dissolution/suspension of the protein, upon orientation on the substrate, and/or during drying of the film.

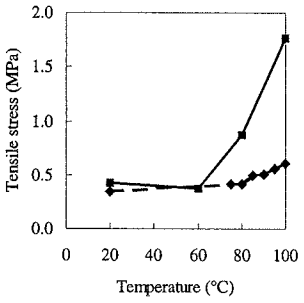

Fig. 3. Tensile strength of wheat gluten films from aqueous suspension depending on the temperature of 10 h thermal treatment on the films (solid line) or 30 min thermal treatment in suspension (dashed line). Films contain 30 wt.% glycerol.

Fig. 2 depicts our hypothesis of protein film formation. In the first step, protein molecules are suspended in water, and a certain degree of destructuration will take place. Upon application onto a substrate, structure changes will occur at the surface (these will probably differ from those in bulk) and the molecules tend to become flatter and more entangled. In the final stage, upon evaporation of water, molecules can become structured once more, engaging into interactions (ionic, hydrophobic) which increase the cohesiveness of the material.

5. Examples on the influence of protein processing on film properties

The influence of temperature treatment on wheat gluten suspensions and films is shown in Fig. 3. The films contained 30% glycerol and were treated for 10 h at a set temperature, and subsequently conditioned at 60% relative humidity at RT. Films that were treated at a temperature in the range of 60–100°C show a significant increase in tensile strength up to a factor of 4. This can be the result of two processes: (1) additional bond formation (crosslinking) in the formed film; and (2) rearrangement of molecules, resulting in more entanglements. Above 65°C, films containing 30% glycerol are sufficiently far above T_G to allow structural mobility. Temperature treatment did influence the tensile strength more than water resistance, indicating an increase in entanglements rather than crosslinks.

Thermal treatment of the suspensions during 30 min does not improve the strength of the final films. Possibly, molecules are slightly crosslinked in suspension but the effect on film properties is negligible. No influence of treatment time was observed between 30 min and 4 h.

Another example of the influence of protein processing, specifically the effect of protein concentration, is the film formation of lysozyme (Engel et al., 1999). In aqueous solution, lysozyme contains 34% α-helix and 18% β-sheet with the

Table 2
Dependence of the appearance and secondary structure of lysozyme films the initial concentration of the lysozyme solution

Initial lysozyme concentration (%)	Predominant secondary structure in film	Film appearance
<0.5 wt.%	β-Sheet	Clear
>2 wt.%	α-Helix	Opaque

remainder being unordered structure. Lyophilization of lysozyme resulted in a decrease of the α-helix content and an increase of β-sheet content (Mishra et al., 1996).

Engel describes that upon lysozyme film formation from aqueous solutions, significant differences appeared in secondary structure and film appearance depending on the initial lysozyme concentration in solution. The results are summarized in Table 2. When casting films from a lysozyme concentration < 0.5% weight, the predominant secondary structure in the dry film was β-sheet, and the films were completely transparent, regardless of the film thickness. Films casted from solutions containing > 2 wt.% lysozyme contained predominantly α-helix (as does the solution) and were opaque. No influence was found on the film thickness and drying time of the films. It seems that the structure changes occur during film formation, though in which stage is a subject of discussion. The results show that water content, even in this low range has a large influence on film properties.

6. Conclusions and open questions

In conclusion it can be stated that from a non-food perspective, denaturation is not recognised as a problem. So far, denaturation occurs during processing, and it even determines processing and properties. A potential could be to develop applications based on (partly) denatured proteins, which could be economically favourable.

Protein processing on the one hand induces denaturation or structural changes, on the other hand these structural changes are a prerequisite for processing, for instance the formation of a cohesive melt, an adhesive or a reactive material. The necessary degree of denaturation is very difficult to assess, and the effect of (the degree of) denaturation on the product properties is little investigated. So far, denaturation was shown to decrease the water uptake of the protein material.

Whether the secondary structure in bulk and on the interfaces/surfaces is significantly different, and how this affects the final properties of the protein product is an open question. And though it is known that denaturation leads to increased reactivity of protein molecules, the effect of denaturation on the efficiency of modification reactions is largely unknown.

(Chemical) modifications are used to adjust protein properties such as solubility, or water resistance. It can be expected that the chemicals used during modification reactions will induce (partial) protein denaturation. This in turn may affect the efficiency of the modification reaction (and of course processing and properties).

In conclusion, the subject of protein denaturation from a non-food perspective represents a major research challenge in the development of technical applications of proteins.

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Heat-induced denaturation and aggregation of ovalbumin at neutral pH described by irreversible first-order kinetics

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Abstract

The heat-induced denaturation kinetics of two different sources of ovalbumin at pH 7 was studied by chromatography and differential scanning calorimetry. The kinetics was found to be independent of protein concentration and salt concentration, but was strongly dependent on temperature. For highly pure ovalbumin, the decrease in nondenatured native protein showed first-order dependence. The activation energy obtained with different techniques varied between 430 and 490 kJ·mole^{-1}. First-order behavior was studied in detail using differential scanning calorimetry. The calorimetric traces were irreversible and highly scan rate-dependent. The shape of the thermograms as well as the scan rate dependence can be explained by assuming that the thermal denaturation takes place according to a simplified kinetic process $N \xrightarrow{k} D$ where N is the native state, D is denatured (or another final state) and k a first-order kinetic constant that changes with temperature, according to the Arrhenius equation. A kinetic model for the temperature-induced denaturation and aggregation of ovalbumin is presented. Commercially obtained ovalbumin was found to contain an intermediate-stable fraction (IS) of about 20% that was unable to form aggregates. The denaturation of this fraction did not satisfy first-order kinetics.

Keywords: Irreversible transitions; scan-rate dependence; scanning calorimetry; chromatography; protein denaturation; aggregation; globular proteins; ovalbumin

Aggregation of proteins is an important process in many biological systems and industrial processes. In biological systems it is required for the assembly of structures with specific functions such as microtubules, blood clots, and viral coatings. The formation of plaques is also related to aggregation of specific proteins that have somehow been modified. The aggregation of proteins is, in general, triggered by a conformational change of the protein induced by heat, enzymatic cleavage, or other processes that affect the folded structure. After this change of structure a series of reactions takes place that lead to the formation of aggregates. In many cases it is not clear what drives the formation of specific structures in these aggregates or the formation of fibrils (Thirumalai et al. 2003). Here we present a study of the heat-induced aggregation of chicken egg white ovalbumin. Ovalbumin is known to form fibrillar types of aggregates upon aggregation and, at high enough protein concentrations, a gel can be formed (Weijers et al. 2002b). It is our aim to use ovalbumin as a model system to study how fibrillar aggregates can be formed and what conditions affect the properties of these aggregates. The results are relevant both to understanding the biological function of pro-

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tein aggregation and for the industrial applications of proteins in food and nonfood systems.

Egg white ovalbumin is a member of the serine protease inhibitor (serpin) superfamily, but shows no recognized protease-inhibitory activity (Stein et al. 1991). Egg white proteins are applied in a wide range of food products. The ability of egg white proteins to form a gel upon heating and their water-binding and emulsifying capacity are important functional properties (Mine 1995). Typical applications are in the meat industry (emulsifier), in foams, in the confectionery industry, and in bakery products (Forsythe 1960; Kiss et al. 1988). For most functional applications, denaturation and aggregation is required. Ovalbumin is the major protein in egg albumin, and much work has been conducted on its thermal aggregation and gelation (Clark et al. 1981; Doi and Kitabatake 1989; Arntfield et al. 1990a,b; Harte et al. 1992; Kitabatake and Kinekawa 1995; Van der Linden and Sagis 2001; Weijers et al. 2002a). It was found by Smith and Back (Smith and Back 1962) that ovalbumin behaves as a mixture of two proteins, where the amount of S-ovalbumin depends on the storage time and pH of the eggs. In the literature, different terminologies are used for ovalbumin. Native (N-) ovalbumin can be converted into Stable (S-) ovalbumin through the formation of an Intermediate (I-). All these species are able to aggregate. The ovalbumin used in this study (SIGMA) contains N-ovalbumin (N_1) and a fraction native ovalbumin (N_2), which can denature, but not aggregate. The DSC profile shows this intermediate fraction, and this fraction is therefore referred as Stable Intermediate (SI-) ovalbumin.

To study the heat-induced denaturation of ovalbumin, we used the model proposed by Lumry and Eyring (1954) for the irreversible denaturation.

$$N_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_1 \xrightarrow{k_2} D_1 \longrightarrow A \qquad (1)$$

Here, N, U, D, and A are native, unfolded, denatured, and aggregated protein forms, respectively, and k_1, k_{-1}, and k_2 are the rate constants for the corresponding reactions. We assume that only D can form aggregates. A commonly used method to study the transition from the native to the denatured state is differential scanning calorimetry (DSC). Equilibrium analysis (N↔U) of DSC thermograms corresponding to reversible unfolding of proteins provides information about the thermodynamics and mechanisms of the reversible unfolding (Privalov 1979, 1982, 1989). However, there are many proteins whose denaturation is irreversible (Donovan and Beardslee 1975; Sanchez-Ruiz et al. 1988; Davoodi et al. 1998; Grinberg et al. 2000; La Rosa et al. 2002), probably due to the occurrence of "side" processes such as aggregation (Klibanov and Ahern 1987). Due to denaturation, hydrophobic interaction can occur, and exposed thiol groups can form disulfide bonds, which results in irreversible behavior (Hoffmann et al. 1996; Alting et al. 2000). Unfolding in the absence of denaturation has been studied using different denaturants (Zemser et al. 1994), but is of less importance in this study, because no kinetic parameters (E_a) can be obtained. Here, thermodynamic parameters such as K will be obtained. Analysis of DSC data for the irreversible denaturation has been reported by several authors (LePock et al. 1992; Kurganov et al. 1997; Lyubarev and Kurganov 2000). In these cases, theoretical equations describing the dependence of the excess heat capacity on temperature were fitted to experimental DSC curves.

Irreversible protein denaturation, as shown in equation 1, involves at least two steps: reversible unfolding of the native protein, followed by the irreversible alteration of the unfolded state to a denatured state, and possibly to another final state. If $k_2 >> k_1$, most of the U molecules will be converted to D (or another final state). The concentration of U will be very low and the amount of U converted into D is restricted to k_1. This results in a simple first-order reaction, the DSC analysis of which has been worked out by Sanchez-Ruiz (Sanchez-Ruiz et al. 1988) using a practical two-state model is represented by:

$$N \xrightarrow{k} D \qquad (2)$$

Gel permeation chromatography is a suitable method to study aggregation kinetics. The aggregation process can be quenched by rapidly cooling the sample to room temperature. Structural properties of the aggregates formed can then be studied. Recently, we reported a detailed study of the heat-induced aggregation and gelation of ovalbumin at low and high ionic strength at neutral pH (Weijers et al. 2002b). As mentioned above, heat-induced aggregation of ovalbumin has been studied intensively by several workers, but none of the earlier investigations on ovalbumin clearly showed the effect of concentration or demonstrated that first-order kinetics applies. Koseki et al. (1989b) found that when a 5-g/L ovalbumin solution in 20 mM potassium-phosphate buffer of pH 7 was heated, the process satisfied first-order kinetics.

In the present study, we investigated the rate of protein conversion for many conditions by varying temperature, protein concentration, and salt concentration. Samples were analyzed by gel-permeation chromatography (GPC). From these results the reaction order, energy of activation (E_a), and the overall rate constants were determined. Subsequently, the kinetic parameters determined by GPC were combined with the kinetic parameters found with DSC, and we propose a new model for the heat-induced denaturation and aggregation of ovalbumin. This model (an extension of the Lumry and Eyring model), which incorporates a

heat-stable fraction, is used to describe both sets of experimental data (protein conversion upon heating and DSC data).

Results

Conversion of monomers into aggregates induced by heating

The heat-induced conversion of ovalbumin monomers to aggregates at neutral pH was measured using two slightly different chromatographic techniques: HPSEC and SEC-MALLS. Temperature, ovalbumin concentration, NaCl concentration, and protein source (WCFS and SIGMA) were varied. The heat-induced aggregates are stable under cooling and dilution, and can thus be characterized at room temperature. As described in Materials and Methods, with HPSEC the samples were acidified to their isoelectric point prior to elution, the denatured and aggregated protein precipitated and the amount of nondenatured monomeric protein left after heating was determined. With SEC-MALLS, the heated protein samples, which were cooled to room temperature, were injected on the column without further treatment. In this case, denatured as well as nondenatured monomers will elute at the same volume, and consequently, no discrimination between denatured and nondenatured monomers can be made. Combining the results determined with both techniques allowed us to properly discriminate nondenatured, denatured monomers, and the aggregated fractions.

Figure 1 shows a chromatogram of heated ovalbumin (WCFS) that was not acidified prior to elution. The ovalbumin solutions were heated at 72°C for different times ranging from 0 to 5000 min. Ovalbumin aggregates elute at volumes between 18 and 23 mL. With increasing heating time, the relatively broad aggregate peak shifts to smaller volumes and the scattered intensity of the aggregate peak increases; this means that the size and amount of the aggregates formed increases. The inset in Figure 1 shows the weight average molar mass of the aggregate peak as calculated from the MALLS data. The molar mass increased from 47 10^3 Da (M of a monomer) for the shortest heating times to $2 \cdot 10^6$ Da (corresponding to aggregates consisting of approximately 45 monomers) for a heating time of 1 10^4 min. The ovalbumin aggregates were always large enough to be clearly distinguished from native proteins. This indicates that under these conditions no or very few stable oligomers (dimers, trimers, etc.) are formed. The size of the aggregates depends on the initial protein concentration and salt concentration during heating, as reported earlier (Weijers et al. 2002b). The polydispersity (M_w/M_n) of the ovalbumin aggregates was approximately 1.5, and did not change significantly as a function of heating time.

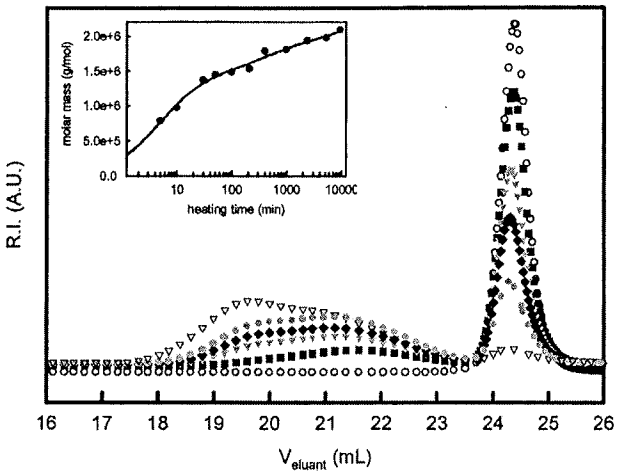

Figure 1. Chromatograms of ovalbumin (WCFS) solutions at pH 7, C = 27.1 gL^{-1}, and 200 ppm NaN_3 at different heating times at 72°C. Heating times: (Open circles) Not heated, (filled squares) 10 min, (filled triangles) 30 min, (filled diamonds) 50 min, (filled circles) 100 min, (open triangles) 5338 min. The *inset* shows the molar mass of the aggregates formed corresponding to the aggregate peaks in the chromatogram.

The narrow peak at volumes between 24 and 25 mL corresponds to nonaggregated monomers. With increasing heating time, the fraction of monomers decreases and stabilizes at about 6% monomers for heating times longer than 1000 min. The size distribution of this fraction was narrow; the polydispersity was about 1.0, and did not increase upon heating.

Comparison of the acidifying and nonacidifying method

The fraction of monomers as a function of heating time, for the acidifying and nonacidifying methods, is shown in Figure 2 for two protein samples (WCFS and SIGMA). Data from acidified and nonacidified heated samples gave significantly different results at long heating times. When SIGMA ovalbumin samples had been subjected to long heating times and subsequently acidified, we found precipitation of all of the monomers, whereas the same samples without acid treatment showed a stable intermediate fraction (SI-ovalbumin) of monomers of approximately 20%. The abundance of this stable fraction did not depend on temperature in the range 72°C to 85°C (data not shown). From the nature of the techniques used, we therefore conclude that this SI-fraction is due to monomers that are denatured but not aggregated. These SI-fractions do not aggregate, and therefore cannot participate in the formation of a network. Consequently, this SI-fraction does not contribute to the structure and gelling functionality. Similar results were obtained for WCFS ovalbumin, but the SI-fraction was smaller, namely approximately 6%.

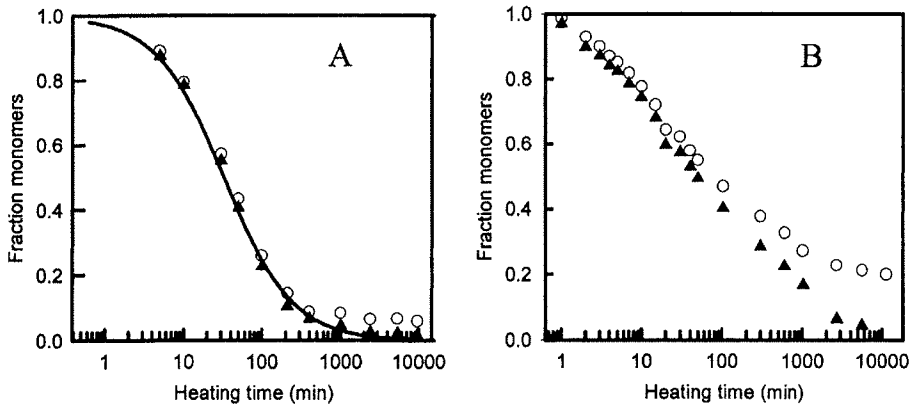

Figure 2. Heating time dependence of the fraction of monomers using the nonacidifying (open circles) and acidifying (filled triangles) method. (*A*) WCFS ovalbumin (pH 7, C = 27 gL^{-1}, T = 72°C), (*B*) SIGMA ovalbumin (pH 7, C = 27 gL^{-1}, T = 78°C). Solid line in *A* represents a fit with reaction order 1.

Dependence of temperature and protein concentration on the denaturation rate

To present a kinetic model for the temperature-induced denaturation and aggregation, a detailed study of the influence of temperature and protein concentration on the denaturation rate was carried out. The relative concentration of nondenatured ovalbumin in the supernatant as a function of heating time, at five temperatures and three initial protein concentrations, is shown in Figure 3A. Table 1 gives an overview of all conditions investigated, for WCFS ovalbumin as well as for SIGMA ovalbumin, and the half time of these reactions. From Figure 3 and Table 1, three interesting observations can be made:

1. A strong temperature dependence on the reaction rate was observed. At 80°C, half of the protein was denatured and aggregated in less than 2 min (half-time, t_h), while at 68.5°C this took approximately 6 h. For SIGMA ovalbumin the effect was even bigger. The rate at which the proteins denatured was strongly temperature dependent, but the shape of the conversion–time curves was the same for all conditions. This is illustrated in Figure 3B, where the data are plotted as a function of time normalized by t_h. Clearly, all data superimpose within experimental error. The data satisfy a denaturation rate with order 1 (solid curve in Fig. 3B). The temperature dependence of the denaturation rate is shown to satisfy Arrhenius' relation (Fig. 4B). An activation energy of about

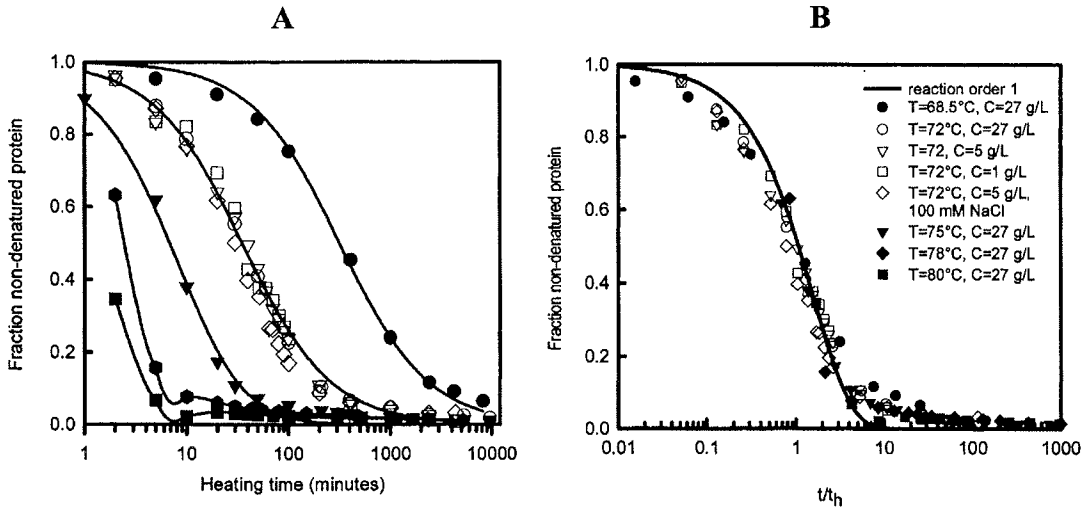

Figure 3. (*A*) Heating time dependence of the fraction of nondenatured ovalbumin (WCFS) under different conditions. (*B*) Same data as in *A* plotted as a function of heating time, normalized by the time needed to denature and aggregate half of the proteins (t_h). Solid lines in *A* are guides to the eye.

Table 1. *Heating conditions of WCFS and SIGMA ovalbumin and the half times (t_h) in which 50% of the protein was denatured and/or aggregated*

Ovalbumin (WCFS)			Ovalbumin (SIGMA)					
Temp. (°C)	Conc. (g/L), added salt	t_h (min)[a]	Temp. (°C)	Conc. (g/L), added salt	t_h (min)[a]	Temp. (°C)	Conc. (g/L), added salt	t_h (min)[a]
80	27, 0 mM	1.2	80	27, 0 mM	1	72	27, 0 mM	126
78	27, 0 mM	2.3 (2.6)	78	27, 0 mM		72	10, 20 mM	125
75	27, 0 mM	7.2 (7.9)	76.5	27, 0 mM	6.7	72	10, 50 mM	127
72	27, 0 mM	37 (39)	76.5	10, 0 mM	5.8	72	10, 100 mM	126
72	5, 0 mM	37 (35)	76.5	5, 0 mM	6.8	70	27, 0 mM	500
72	5, 100 mM	37 (37)	76.5	1, 0 mM	6.4			
72	1, 0 mM	37 (43)	74.8	27, 0 mM	48			
68.5	27, 0 mM	320 (306)	72	60, 0 mM	126			

[a] t_h determined from Figure 3, between brackets the value of t_h calculated from first order kinetics (ln 2/k) as shown in Figure 4A.

480 kJ·mole^{-1} was found. This is comparable to the activation energy found for other proteins, such as β-lactoglobulin (Le Bon et al. 1999), actin, carboxypeptidase, creatine kinase, etc. (Lyubarev and Kurganov 2000). The large value of E_a might be expected, because the highly cooperative nature of the protein implies a large Δh between the folded and denatured protein, and E_a always larger than Δh (see also Le Bon et al. 1999).

2. The half-time of the reaction, as described in Table 1, did not depend on initial protein concentration (within the experimental range of 0.1–60 g/L; Fig. 5). Also, no dependence of the ionic strength on the half time was found (in the range of 3–100 mM). This is different from results found for β-lactoglobulin; here, the decrease in concentration of native β-lactoglobulin during heating was fitted by a reaction order of 1.5 (Roefs and De Kruif 1994; Le Bon et al. 1999). For comparison, the concentration dependence of the half-time of β-lactoglobulin is also plotted in Figure 5 (data taken from Le Bon et al. 1999). From the data presented in Figure 5, they found for β-lactoglobulin a total reaction order of 1.5. From the results so far, it is clear that the decrease of nondenatured protein as a function of heating time only depends on temperature and neither on protein concentration nor on NaCl concentration, which suggests that a unimolecular process like denaturation is the rate-limiting step in this process.

3. Finally, remarkable differences in denaturation rates were observed between the two sources of ovalbumin. SIGMA ovalbumin denatures much more slowly than WCFS ovalbumin. This may be due to the storage time of the eggs before purification or to the purification itself, and will be discussed in the general discussion section.

Differential scanning calorimetry of ovalbumin under irreversible conditions

Differences between WCFS and SIGMA ovalbumin were also observed with DSC. Figure 6 shows the DSC thermo-

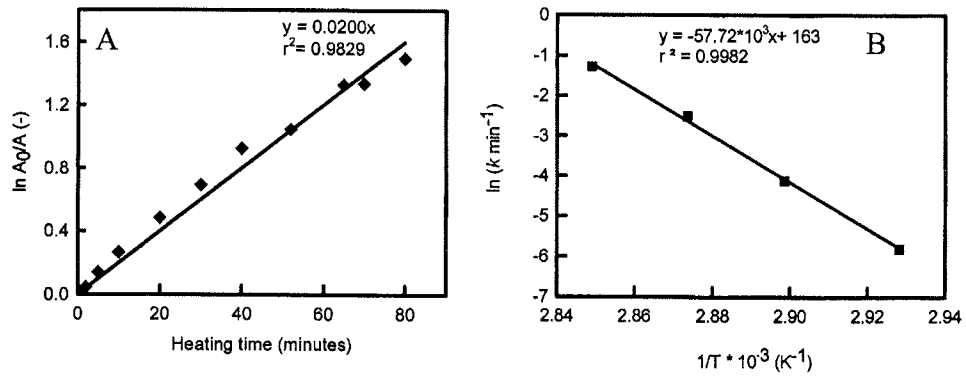

Figure 4. (*A*) The fraction of nondenatured ovalbumin (WCFS) (A_0 = 5 g/L, [NaCl] = 100 mM, T = 72°C) as a function of heating time shows a first-order dependence. The half-time (t_h) is calculated from ln 2/k, where k is a temperature-dependent rate constant, obtained from the slope. (*B*) Arrhenius plot obtained from data from Figure 3A, which are analyzed as in Figure 4A.

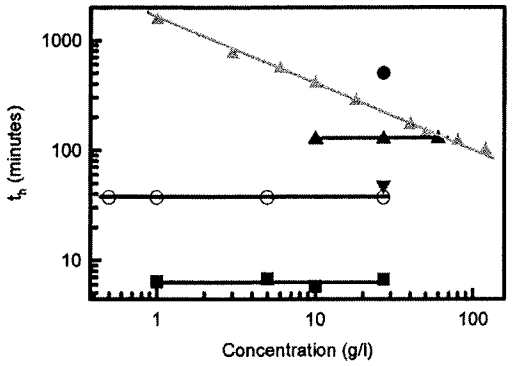

Figure 5. Concentration dependence of the half-time for ovalbumin and β-lactoglobulin (data β-lactoglobulin taken from Le Bon et al. 1999). Black symbols represent SIGMA ovalbumin. Open symbols represent WCFS ovalbumin, and gray symbols represent β-lactoglobulin. Heating conditions: (Black circles) T = 70°C, (black triangles) T = 72°C, (black upside-down triangles) T = 75°C, (black squares) T = 76.5°C, (open circles) T = 72°C, (gray triangles) T = 67°C.

grams of both ovalbumin sources, at a scan rate of 0.5°C min^{-1}. For both ovalbumin sources the calorimetric transitions were apparently irreversible. After cooling from the first run, no transition could be detected in the second heating run. The ovalbumin obtained from SIGMA shows two overlapping endothermic peaks centered at 78.4°C (T_{p1}) and 82.45°C (T_{p2}), respectively. The second peak represents a stable intermediate (SI), which is an intermediate in the conversion of native ovalbumin (N) into stable ovalbumin (S). The presence of this intermediate has been reported by others (Huntington et al. 1995; Hagolle et al. 1997). The appearance of two distinct peaks in the thermogram of SIGMA ovalbumin indicates that no rapid conversion between the two native states is possible, and therefore, both fractions denature independently. A fast equilibrium between the two fractions (N_1 and N_2) would imply a fixed ratio between N_1 and N_2. Therefore, the ratio between dD_1/dt and dD_2/dt would also be fixed, and no calorimetric distinction could be made between the two fractions. Barbut and Findlay have shown that when ovalbumin and its stable form (S-ovalbumin) are both present in a solution, they undergo denaturation independently.

From DSC experiments (Fig. 6), the presence of an SI-fraction of about 20%, as observed with SEC-MALLS, is confirmed. We suggest that the decrease in denaturation rate of SIGMA ovalbumin is probably due to the interference of a 20% SI-fraction (intermediate between N-ovalbumin and S-ovalbumin), which can denature but cannot aggregate. Lumrey and Eyring (1954) originally proposed the two-state kinetic model. Based on our findings, we propose an irreversible two-state kinetic model for the denaturation and aggregation of ovalbumin in general.

$$\begin{array}{l} N_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_1 \xrightarrow{k_3} D_1 \longrightarrow A \\ \qquad k_{-5} \uparrow\downarrow k_5 \\ N_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} U_2 \xrightarrow{k_4} D_2 \end{array} \qquad (3)$$

In our model, we assume two different native states in the starting material; both native states denature independently upon heating. There is no fast equilibrium between N_1 and N_2. Denaturation is the rate-limiting step in this process, which means that when the protein unfolds (and denatures), one of the denatured states (D_1) aggregates immediately, while the other one cannot aggregate and is therefore referred to as the stable fraction (D_2).

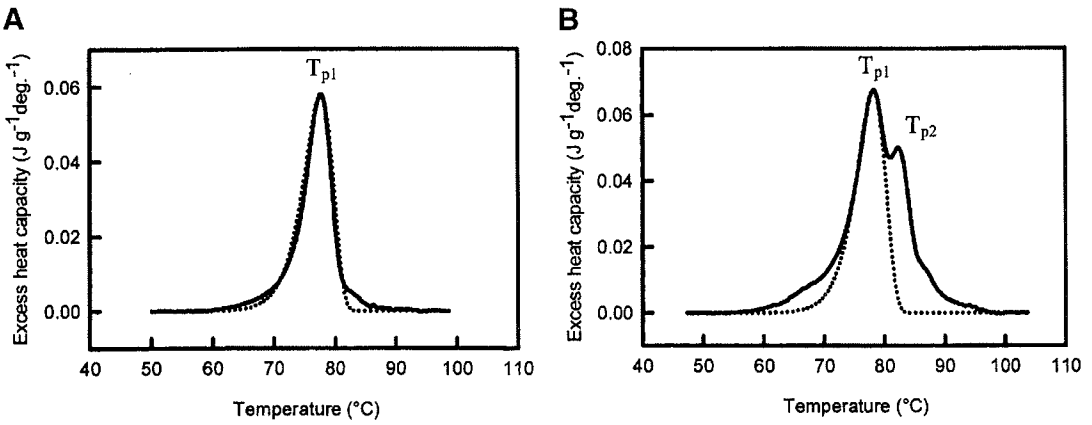

Figure 6. Differential scanning calorimetry thermograms and the best fit of the two-state irreversible denaturation model (χ^2 of $4 \cdot 10^{-6}$) of WCFS ovalbumin (*A*) and SIGMA ovalbumin (*B*) solutions at a scan rate of 0.5°C/min (solid lines) and pH 7.0, protein concentrations of 20 g/L and 35 g/L, respectively. Dashed lines represent irreversible first-order fits.

As we show below, our irreversible two-state kinetic model, based on a mixture of two different ovalbumin fractions, is supported by DSC measurements.

From detailed DSC experiments we attempted to get answers to the following questions: (1) What is the activation energy of both sources of ovalbumin, and are these values comparable with the values found for E_a with other techniques as described above? (2) Can we describe experimental DSC thermograms of WCFS and SIGMA ovalbumin with irreversible first-order kinetics? (3) Is it possible to describe experimental data for the conversion of nondenatured monomers into aggregates with two first-order rate constants, as we assume in our model?

Activation energy of ovalbumin

The activation energy for the denaturation of ovalbumin can be obtained from the scan rate dependence of the peak temperature from a DSC thermogram. Table 2 gives the temperatures corresponding to the maximum in heat capacity (T_p) at different scan rates for both ovalbumin sources. The results presented in Table 2 clearly show a strong dependence on the scan rate, which indicates the absence of equilibrium between native and the denatured state, during scanning.

To check whether the results are in agreement with the proposed first-order reactions, we used the model developed by Sanshez-Ruiz et al. (1988). Briefly, the two-state model can be represented as $N \xrightarrow{k} D$, where N is the native state, D is the unfolded state or, more probably, a final state, with k being a first-order rate constant. From the following equation the energy of activation can be calculated from DSC data.

$$\frac{v}{T_p^2} = \frac{AR}{E} e^{-E_a/RT_p} \quad (4)$$

where v is the scan rate, E_a the energy of activation, A the frequency factor of the Arrhenius equation, and R the gas constant.

Table 2. *Effect of scan rate on peak temperatures (T_p) for WCFS ovalbumin (C = 10 g/L) and SIGMA ovalbumin solutions (C = 20 g/L)*

Ovalbumin (WCFS)		Ovalbumin (SIGMA)		
Scan rate (°C/min)	T_{p1} (°C)	Scan rate (°C/min)	T_{p1} (°C)	T_{p2} (°C)
0.10	74.81	0.05	X	78.22
0.20	75.40	0.10	75.72	79.41
0.30	76.66	0.20	76.62	80.78
0.50	77.72	0.50	78.38	82.45
1.00	79.04	1.00	79.86	83.46
1.50	79.71	1.50	80.66	83.31
2.00	80.82	2.00	81.34	83.95

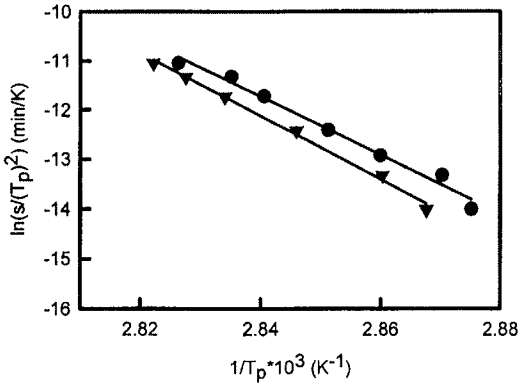

Figure 7. Plots of ln (v/T_p^2) versus $1/T_p$ for WCFS (filled circles) and SIGMA (filled triangles) ovalbumin corresponding to equation 2 in the text. Each data point corresponds to a scan rate given in Table 2.

If the proposed model is correct, equation 4 requires a linear dependence of ln (v/T_p^2) versus $1/T_p$. Indeed, a linear dependence is observed (see Fig. 7). The E_a calculated from the scan rate dependence was found to be 490 kJ·mole^{-1} for WCFS ovalbumin, which is in very good agreement with values found with other techniques as reported in Table 3. The energy of activation obtained from the scan rate dependence for SIGMA ovalbumin is obtained for the main fraction. The scan rate dependence of the main peak is almost identical to the WCFS one with an E_a of 530 kJ·mole^{-1}. The E_a of the additional fraction (T_{p2}) has not been calculated, because it is doubtful from HPSEC and DSC analysis whether this fraction shows first-order behavior. Using the E_a and A obtained from experimental data of WCFS and SIGMA (T_{p1}) ovalbumin, the rate constants are calculated at different temperatures (Fig. 8). From Figure 8 it is clear that the rate constants for WCFS and the main fraction of SIGMA ovalbumin are almost identical. This indicates that the main fraction of SIGMA is probably the same as the WCFS ovalbumin and that both can be described with first-order kinetics.

Fitting thermograms of ovalbumin with irreversible first-order kinetics

Figure 6 shows the best fit of the experimental DSC profile (including a baseline correction, resulting in $\Delta C_p = 0$ Jg^{-1}K^{-1}) recorded at 0.5°C/min, for WCFS and SIGMA ovalbumin. It is evident that the two-state irreversible denaturation (N→D) model reproduces the experimental data well in the whole temperature range for WCFS ovalbumin. The parameters obtained from the best fit are: E_a = 430 kJ·mole^{-1}; T_{p1} = 77.68°C using the condition that the surface area under the peak is the same: $\Delta h \approx 800$ kJ·mole^{-1}. Note that DSC measures the Δh of the overall process, while kinetics deals only with the rate-determining step. This re-

Table 3. *Activation energy (E_a) and frequency factor (A) of the Arrhenius equation determined from experimental DSC and HPGPC data and the best fit (thermograms) for WCFS and SIGMA ovalbumin*

	HPGPC		DSC		DSC FIT
	E_a (kJ · mole^{-1})	A (–)	E_a (kJ · mole^{-1})	A (–)	E_a (kJ · mole^{-1})
WCFS	480	6.17×10^{70}	492	5.17×10^{72}	430
SIGMA (T_{p1})			530	1.67×10^{78}	430

sult is an additional indication of a multistep reaction. Attempts were also made to fit the experimental data for SIGMA ovalbumin by the two-state irreversible denaturation model. The main peak is successfully generated with almost the same parameters as for WCFS ovalbumin: $E_a = 430$ kJ·mole^{-1}; $T_{p1} = 78.28$°C and $\Delta h \approx 800$ kJ·mole^{-1}, where the fraction participating in the main peak was 81% (SI-fraction 19%). We neglected the minor shift of the peak due to the additional fraction. However, the best fit of the additional peak was less successful. A too high E_a had to be used to fit the peak height, and this resulted in a too small peak. From this result we conclude that the denaturation of the additional fraction cannot be described with irreversible first-order kinetics.

Experimental data described with two first-order rate constants

For the determination of the rate of decrease of the nondenatured ovalbumin concentration upon heating, chromatography experiments were carried out. From Figure 4A, it is clear that experimental data for WCFS ovalbumin show first-order behavior. Experimental data of SIGMA ovalbumin could not be described with first-order dependence because this ovalbumin contained two fractions. Therefore, we attempted to describe the experimental data with two first-order rate constants (equation 5)

$$\frac{A}{A_0} = (1-f)e^{-k_1 t} + fe^{k_2 t} \qquad (5)$$

where A/A_0 is the fraction of denatured protein, and f the fraction of denatured but not aggregated protein ($f = 20\%$). Fitting the experimental data assuming two first-order rate constants was not successful, because, again, the additional fraction probably does not denature by a first-order process.

Discussion

We investigated the effect of temperature, protein concentration, and salt concentration on the protein consumption of ovalbumin. From the experimental results a kinetic model for the temperature-induced denaturation and aggregation of ovalbumin is presented.

We observed that t_h was not dependent on protein concentration, nor on salt concentration. For WCFS ovalbumin, first-order kinetics was observed as shown in Figure 4A. Note that for long heating times interference of the 6% SI-ovalbumin occurs. This results at long heating times in a deviation from first-order kinetics. For SIGMA ovalbumin first-order dependence could not be obtained because of an additional fraction of 20% SI-ovalbumin. Combining the acidifying and nonacidifying techniques, we found that this additional SI-fraction is able to denature, but cannot aggregate. Two different ways to obtain heat-stable ovalbumin have been suggested (see thermograms, Fig. 6). (1) The amount of S-ovalbumin is related to the storage time and pH of the eggs. Increased storage time results in a growth of the intermediate fraction (Smith and Back 1962). We found that ovalbumin prepared from different batches of eggs resulted in different amounts of SI-ovalbumin, and therefore, different kinetic behavior was observed (data not shown). (2) The formation of I-ovalbumin could be induced by the incubation of purified ovalbumin at basic pH (9.9) and elevated

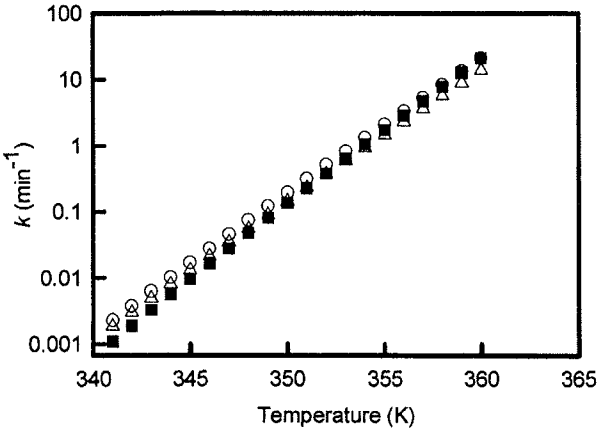

Figure 8. Rate constant (k) for WCFS and SIGMA ovalbumin as a function of temperature. First-order rate constant changes with temperature as described in the Arrhenius equation; frequency factor and energy of activation are obtained from Table 3. Rate constants calculated for (open circles) WCFS (DSC), (open triangles) WCFS (HPGPC), (filled squares) SIGMA (T_{p1}).

temperature (55°). It has been demonstrated that by varying the incubation time, ovalbumin is converted in time into more heat-stable forms like I- and S-ovalbumin. (J. De Groot and H. H. J. De Jongh, private commun). In the latter case, I- and S-ovalbumin can form aggregates, whereas we find here that it is not possible for our 20% fraction SI-ovalbumin to form aggregates. Therefore, it is most likely that the fraction SI-ovalbumin, present in the SIGMA batch, is formed in the egg as mentioned under (1).

The presence of 20% SI-ovalbumin was also confirmed by DSC, where we clearly observed that SIGMA ovalbumin behaved as a mixture of two proteins with different rates of denaturation. The thermograms for the thermal denaturation of ovalbumin can be interpreted in terms of a kinetic process $N \xrightarrow{k} D$, where k is a first-order kinetic constant that changes with temperature as described by the Arrhenius equation. This model predicts that the peak positions should be dependent on the scan rate, as is the case for ovalbumin. For SIGMA ovalbumin the scan rate dependence is used for the main peak, where we neglected the minor shift of the peak due to the additional fraction. The above-mentioned simple two-state model appears to imply that the denatured state is thermodynamically more stable than the native one. A more realistic representation of the irreversible thermal denaturation of ovalbumin, which results in the same first-order kinetic process, is probably given by:

$$\begin{array}{ccccc} & & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & \overset{k_3}{\longrightarrow} & \\ N_1 & & U_1 & & D_1 \longrightarrow A \\ & k_{-5} \uparrow\downarrow k_5 & & & \\ & & \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} & \overset{k_4}{\longrightarrow} & \\ N_2 & & U_2 & & D_2 \end{array} \qquad (3)$$

where N_1 and N_2 are the native states, U_1 and U_2 the unfolded states, D_1 and D_2 the denatured states, and A a final state, which is in this case an aggregated form. If we assume that the conversions of N to U are first-order processes and $k_{3,4} >> k_{1,2}$, most of the U molecules will be converted into A instead of returning to N through the process U→N. As a result no equilibrium between N and U will be established, so that the denaturation may be considered as being an irreversible process N→A, kinetically controlled by a slow conversion of N→U. This is valid for WCFS ovalbumin; indeed, the DSC traces could be well described with this model resulting in a χ^2 of $4 \cdot 10^{-6}$. Calculations on the same DSC traces (given concentration, Δh and T_p) using a reversible process resulted in a χ^2 of $5 \cdot 10^{-5}$. The authors interpret the results of the fit as extra support for the proposed model.

For SIGMA ovalbumin the situation is more complex. If we assume two native fractions (as was confirmed by DSC and chromatography) and $k_{5,-5} >> k_{1,2}$, then a fast equilibrium between N_1 and N_2 is established. This would imply a fixed ratio between N_1 and N_2, and therefore, no calorimetric distinction between the two can be made. However, this is not applicable to SIGMA ovalbumin, because two peaks are observed. If $k_{5,-5} << k_{1,2}$, N_1 and N_2 denature independently, and this will result in a calorimetric distinction between the two. The DSC traces should be consistent with two first-order reactions. For SIGMA ovalbumin it was clear that N_1 could be fitted with the same parameters as for WCFS ovalbumin, while N_2 could not be fitted with irreversible first-order kinetics at all. Therefore, we conclude that N_2 probably does not denature by a first-order process. A possibility is that $k_{5,-5} \sim k_1$ ($k_1 \sim 0.3$ min^{-1}). For that case, and under specific conditions, numerical calculations showed that N_1 could be described with irreversible first-order kinetics, whereas N_2 could not. We must emphasize that the latter ideas are rather speculative.

Conclusions

The rate of decrease of nondenatured native WCFS ovalbumin when heating solutions of ovalbumin at pH 7 can be described with first-order kinetics, which means that denaturation is the rate-limiting step. The temperature dependence obeys Arrhenius law and is controlled by an activation energy of about 490 kJ·mole^{-1}. From analyzing the shape of the thermograms as well as the scan rate dependence, activation energies of respectively 430 and 490 kJ·mole^{-1} are found. In contrast to WCFS ovalbumin, SIGMA ovalbumin showed two distinct peaks. The main peak could be successfully described with the same parameters as for WCFS ovalbumin, and assuming 81% of the protein participating in the main peak. Analyzing the scan rate dependence of the main fraction, an activation energy of 530 kJ·mole^{-1} was found, in good agreement with values found for WCFS ovalbumin. However, experimental results cannot be sufficiently described by implementing a second first-order process (equation 5) corresponding to the additional fraction in SIGMA ovalbumin. Therefore, we conclude that the denaturation of the additional fraction (SI-ovalbumin) does not behave as a first-order process.

Materials and methods

Ovalbumin

Two different sources of hen egg ovalbumin were used in this study. Ovalbumin was purified from egg white of freshly laid hens; eggs (less than 2 h) based on the procedure of Vachier et al. (1995). In the text, this source of pure ovalbumin is referred to "WCFS-ovalbumin". Another source of hen egg ovalbumin was purchased from Sigma (grade V, >99% pure by agarose electrophoresis, crystallized and lyophilized, Lot # 19H7002). This type of ovalbumin is referred in the text as SIGMA ovalbumin. The purity of both sources was checked with mass spectrometry and

SDS PAGE. The latter showed for WCFS ovalbumin one clear spot at 45 kD and for Sigma ovalbumin an additional very small spot was observed at about 80 kD (data not shown). Mass spectrometry was not possible for ovalbumin because it showed many envelopes since it contains 2, 1, and 0 phosphate groups per molecule and is usually glycosylated.

For kinetic experiments, ovalbumin (both WCFS and SIGMA) was dissolved in double-distilled water with 3 mM NaN_3 added to avoid bacterial growth. Final protein concentrations were varied between 0.1 and 60 g/L and NaCl concentrations between 0 and 100 mM. The solutions were subsequently stirred for at least 2 h at ambient temperature to allow the protein to dissolve. The pH of the solution was adjusted to 7.0 and the solution was subsequently centrifuged and filtered (0.45 μm; Millex-SV, Millipore Corp., Bedford, MA) to remove a small fraction of insoluble material. The protein solutions were heated in screw-cap vials containing ca. 5 mL of ovalbumin solution in a temperature-controlled water bath at temperatures varying between 68.5 and 80°C. After heating, the solutions were rapidly cooled by placing the tubes in ice-water. The amount of nondenatured native proteins after heat treatment was determined with a standard assay involving acid precipitation and high-performance size-exclusion chromatography (HPSEC; Hoffmann and Mil 1997). The total amount of monomeric protein (nondenatured as well as denatured) after heat treatment was determined with a standard assay without acidification step, employing size-exclusion chromatography in combination with multiangle laser light scattering (SECMALLS).

For differential scanning calorimetry (DSC) experiments, ovalbumin solutions (WCFS) of 1, 5, 10, and 20 g/L without added NaCl were prepared as described above. For ovalbumin solutions (SIGMA), concentrations of 20 and 35 g/L without added NaCl were used.

HPSEC

For the determination of the rate of decrease in concentration of nondenatured ovalbumin upon heating, different series of screw-cap vials containing ca. 5 mL of ovalbumin solution with different initial concentrations were heated at 80, 78, 75, 72, and 68.5°C for WCFS ovalbumin, and 80, 78, 76.5 75, 72, and 70°C for SIGMA ovalbumin. The vials were cooled in ice-water and the protein solutions were diluted to a final concentration of nondenatured protein in the range of 0.1 to 5 g/L, to be within the calibration range. Then, the pH was adjusted to 4.7 ± 0.1 with 0.1 M HCl, which causes the denatured and aggregated ovalbumin to precipitate. After centrifugation at 20,000*g* for 5 min at ambient temperature, the concentration of nondenatured ovalbumin in the supernatant was determined by HPSEC (Phenomenex BioSep-SEC-S2000 column, 300 × 7.5 mm) with UV detection at 280 nm. In this report, this technique is referred to the "acidification" method, and is assumed to measure the amount of nondenatured native protein left after heating.

SECMALLS

Heated ovalbumin solutions (without acid treatment) were injected after cooling into a high-performance gel chromatography system consisting of a PL-GFC 300, PL-GFC 1000, and PL-GFC 4000 column (300 × 7.5 mm) in series (Polymer Laboratories Ltd.). The specified exclusion limits of these three columns are $1{\cdot}10^5$, $2{\cdot}10^6$, and $1{\cdot}10^7$ Daltons, respectively. The eluant was a 25 mM TRIS/Tricine buffer, pH 8.0, the flow rate was 1.0 mL/min. Heated ovalbumin samples were diluted with double-distilled water to a protein concentration of approximately 10 g/L. These samples were subsequently diluted with twofold concentrated eluant (50 mM TRIS/Tricine), after which 200-μL aliquots were injected into the chromatographic system. This technique is referred in this report as the "nonacidification" method; it determines the total amount of monomeric protein (both native and denatured).

For on-line light-scattering detection a DAWN-F MALLS photometer (Wyatt Technology) was used, equipped with a K5 flow cell and a linearly polarized He-Ne laser light source (5 mW) with a wavelength of 632.8 nm. The DAWN contains 18 detectors, but the four smallest scattering angles (θ) were excluded, as the signal-to-noise ratio of these detectors was too low for an accurate measurement. The concentration of the eluting material was determined with a UV spectrophotometer (Jasco CD-1595, Japan) at 280 nm and a differential refractometer (ERC-7510 ERMA Optical Works Ltd). The data were accumulated and processed using Astra for Windows, version 4.0. The molar mass M_i of the ovalbumin monomers and aggregates in each fraction i was calculated with a first-order Debye fit, using a specific refractive index increment (dn/dc) of 0.172 cm^3/g for ovalbumin in TRIS/Tricine buffer.

DSC

DSC scans were carried out with a SETARAM micro-DSC III with stainless steel 1-mL sample cells. Calibration was done with naphthalene. The DSC has two measuring cells: one is used for the sample, and the other, for the reference. Samples were prepared at ovalbumin (WCFS) concentrations of 5, 10, and 20 g/L, pH 7.0, without added salt. For ovalbumin (SIGMA) concentrations of 20 and 35 g/L were used. The same solution without the protein was used in the reference cell. The temperature was scanned from 25 to 120°C at scanning rates of 0.05, 0.10, 0.20, 0.30, 0.50, 1.00, 1.50, 2.00, and 3.00°C min^{-1}. To obtain the C_p^{eff} curves, reference–reference baselines were obtained at the same scanning rate and subtracted from the sample curves.

For DSC scans at an ovalbumin (WCFS) concentration of 1 g/L, a Microcal MC-2 calorimeter was used. Experiments with varying protein concentrations in the range of 1–10 gL have shown that DSC transitions do not depend on protein concentration (data not shown).

The statistics of the fit are calculated using the following equation:

$$\chi^2 = \frac{1}{n^{eff} - p} \sum [y_i - f(x_i; p_1, p_2, \ldots)]^2 \qquad (6)$$

where n^{eff} are the number of data points and p the number of parameters.

Acknowledgments

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